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# Electron microscopic investigation of the morphology and calcium-induced fusion of lipid vesicles with an oligomerised inner leaflet

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## Abstract

The lipid head groups in the inner leaflet of unilamellar bilayer vesicles of the synthetic lipids DHPBNS and DDPBNS can be selectively oligomerised. Earlier studies have established that these vesicles fuse much slower and less extensively upon oligomerisation of the lipid head groups. The morphology and calcium-induced fusion of vesicles of DHPBNS and DDPBNS were investigated using cryo-electron microscopy. DHPBNS vesicles are not spherical but flattened, ellipsoidal structures. Upon addition of  $\text{CaCl}_2$ , DHPBNS vesicles with an oligomerised inner leaflet were occasionally observed in an arrested hemifused state. However, the evidence for hemifusion is not equivocal due to potential artefacts of sample preparation. DDPBNS vesicles show the expected spherical morphology. Upon addition of excess  $\text{CaCl}_2$ , DDPBNS vesicles fuse into dense aggregates that show a regular spacing corresponding to the bilayer width. Upon addition of EDTA, the aggregates readily disperse into large unilamellar vesicles. At low concentration of calcium ion, DDPBNS vesicles with an oligomerised inner leaflet form small multilamellar aggregates, in which a spacing corresponding to the bilayer width appears. Addition of excess EDTA results in slow dispersal of the  $\text{Ca}^{2+}$ -lipid aggregates into a heterogeneous mixture of bilamellar, spherical vesicles and networks of thread-like vesicles. These lipid bilayer rearrangements are discussed within the context of shape transformations and fusion of lipid membranes. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Vesicles; Polymerisable lipids; Cryo-electron microscopy; Membrane fusion; Calcium ion

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## 1. Introduction

Structural characterisation of the lipid bilayer rearrangements in the course of membrane fusion is a long-standing challenge to electron microscopists. The main obstacle is the inherent transient and local nature of the fusion event. In addition, microscopic studies are frustrated by potential artefacts that arise during sample preparation. Staining, dehydration, vitrification, fracturing, and shear during blotting and film formation, all pose risks to the fragile lipid structures that may occur during membrane fusion (Burger et al., 1993).

Meanwhile, the views about the molecular rearrangements that occur when two bilayer membranes merge into one have converged to the widely accepted 'stalk-pore' model (Siegel, 1993, 1999; Chernomordik and Zimmerberg, 1995). Briefly, it is assumed that two adjacent bilayers are brought into close contact, e.g. because anchoring proteins pull them together, or upon binding of fusogenic ions that expel hydration water and eliminate electrostatic repulsion, or by polymer-induced depletion. Close approximation is accompanied by local disruption of the bilayer structure, leading to the transient establishment of a stalk-like structure. The stalk is believed to expand into a hemifusion intermediate in which the two fusing compartments are separated by one mutual bilayer membrane, the 'bilayer diaphragm'. Consequently, it is proposed that as the diaphragm widens, a minute hole forms in it, leading to the formation of a fusion pore of limited size. Pore formation is thought to be reversible up to a certain size, beyond which the pore irreversibly opens and full fusion is achieved. This model of membrane fusion is supported by a large amount of sound data from fluorescence studies and membrane capacitance and conductance measurements. The sequence of events during bilayer fusion (Lee and Lentz, 1997), the sensitivity of the kinetics of formation of the various intermediate states to changes in the spontaneous curvature of either of the two bilayer leaflets (Chernomordik et al., 1993, 1995a,b; Melikyan et al., 1997), and the reversibility of hemifusion and pore formation (Nanavati et al., 1992;

Chanturiya et al., 1997; Lee and Lentz, 1997) have been demonstrated.

The first micrographs of fusing membranes were obtained from freeze-fracture and thin-section electron microscopy (EM) of zoospores (Pinto da Silva and Noguira, 1977). In fact, this early report is the first to mention the hemifusion diaphragm as a potential fusion intermediate. Fusion pores were observed in freeze-fracture EM of granules in mast cells (Chandler and Heuser, 1980). Calcium-induced fusion of phosphatidylserine vesicles was studied by freeze-fracture EM (Papahadjopoulos et al., 1975; Kachar et al., 1986). 'Point defects' and 'lipidic intramembranous particles' (IMPs), indicative of primary contact sites of adjacent bilayers that may be on the verge of fusion, were observed in freeze-fracture EM studies of concentrated lipid suspensions containing substantial amounts of phosphatidylethanolamines (Hui et al., 1981; Verkleij, 1984). Generally, these 'particles' were viewed as so-called 'inverted micellar intermediates' (IMIs) that were believed to constitute the onset of lamellar-to-inverted-hexagonal ( $L_{\alpha}$ - $H_{II}$ ) phase transitions. Based on theoretical considerations (Siegel, 1993, 1999), the search for inverted micellar intermediates was abandoned in favour of a quest for stalk-like structures and early fusion pores or 'interlamellar attachments' (ILAs). Again, formation of stalks and ILAs was associated with lamellar-to-inverted-hexagonal phase transitions, which were characterised in cryo-EM studies of more dilute liposome solutions (Frederik et al., 1989, 1991; Siegel et al., 1989, 1994; Siegel and Epand, 1997). Recently, membrane microp protrusions as well as the initial fusion pores of influenza virus-liposome fusion were revealed in freeze-fracture EM of pelleted virus/liposome mixtures (Kanaseki et al., 1997). Most of the studies mentioned above suffered from the inherent transient nature of the fusion event: all intermediate stages of membrane fusion have limited life-times, they are localised events that occur infrequently, and observations are essentially a matter of lucky shots. In many systems attempts were made to circumvent this problem by a drastic increase in lipid concentration.

The amphiphilic compounds DDPBNS and DHPBNS are members of a family of synthetic lipids that provides a useful membrane mimetic system (Ravoo et al., 1996, 1998, 1999, 2000). The molecular structures of DDPBNS and DHPBNS are presented in Fig. 1. DDPBNS has two dodecyl hydrocarbon chains and a main phase transition temperature ( $T_m$ ) of  $-1^\circ\text{C}$ . DHPBNS has two hexadecyl hydrocarbon chains and a  $T_m$  of  $40^\circ\text{C}$ . The  $\beta$ -nitrostyrene head groups of the lipids form linear oligomers upon UV irradiation (Ravoo et al., 1996; Ravoo, 1998). Following selective hydrolysis of the  $\beta$ -nitrostyrene groups in the exovesicular bilayer leaflet, the  $\beta$ -nitrostyrene groups in the inner leaflet can be photopolymerised, yielding vesicles with an oligomerised inner leaflet (Ravoo et al., 1996).  $^{31}\text{P}$ -NMR spectroscopy, permeability studies, and detergent resistance measurements indicated that the head groups have a reduced mobility and pack more closely upon oligomerisation (Ravoo et al., 1999). DSC showed that the main phase transition temperature (indicative for the flexibility of the alkyl chains in the bilayer interior) is unaffected, although the phase transition occurs less cooperatively in the oligomerised bilayer (Ravoo, 1998). It was shown by a combination of lipid mixing assays — light scattering and electron microscopy that vesicles of DDPBNS and DHPBNS (and structurally related lipids) undergo efficient calcium-induced fusion (Ravoo et al., 1999). However, after oligomerisation of the lipid head groups, the vesicles aggregate upon addition of calcium ion, but

calcium-induced fusion is strongly inhibited due to a combination of reduced lipid lateral mobility and bilayer curvature effects (Ravoo et al., 1999). This membrane model system was exploited in a microcalorimetric study to determine the enthalpies associated with the consecutive elementary stages of (1) binding of calcium ion, (2) vesicle aggregation, (3) and bilayer fusion in the course of calcium-induced vesicle fusion (Ravoo et al., 1998). It was found that fusion of small vesicles is associated with a small positive enthalpy and requires an entropic driving force. Furthermore, the fusion of vesicles of DHPBNS with Sendai virus was investigated (Ravoo et al., 2000). It was observed that vesicle-virus fusion is strongly inhibited by lipid head group oligomerisation. According to a microcalorimetric analysis, vesicle-virus fusion is associated with a much larger positive enthalpy than vesicle-vesicle fusion.

The work described in this report stems from the assumption that the oligomerisable lipid vesicles present a promising avenue towards structural characterisation of intermediate structures of membrane fusion. Bilayer vesicles of  $\beta$ -nitrostyrene lipids such as DDPBNS and DHPBNS can be oligomerised exclusively in their inner leaflet, resulting in membranes with an extreme transverse asymmetry: while the outer leaflet is fusogenic, the inner leaflet is not. The stalk-pore model of membrane fusion predicts that extensive hemifusion occurs between such bilayers, whereas full fusion will be strongly inhibited. However, the stalk-pore model of membrane fusion is based on

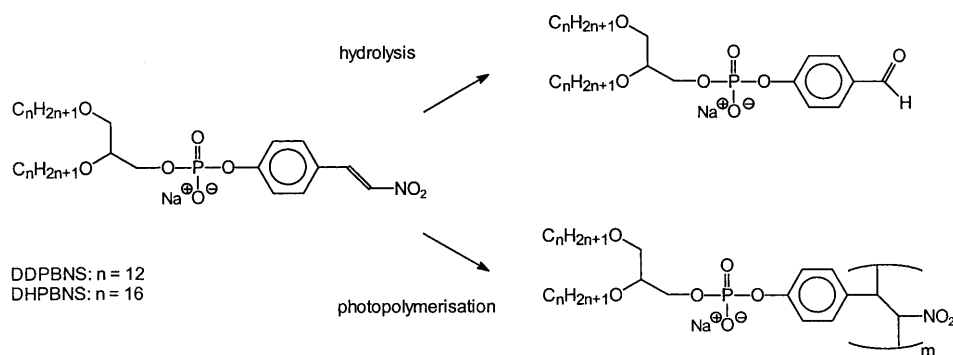


Fig. 1. Molecular structures of the synthetic lipids DDPBNS and DHPBNS, and the products that result from hydrolysis and photopolymerisation.

considerations of hydrophobic attractions and curvature stress, but does not take into account electrostatic interactions and (de)hydration effects (Siegel, 1993; Siegel, 1999). Therefore, it is not certain that this model provides an accurate description of calcium-induced bilayer fusion. Calcium-induced fusion of small vesicles of phosphatidylserine has been described in terms of a so-called ‘rupture-reseal’ mechanism, in which the small vesicles initially fuse into large lamellar sheets, which roll up into multilamellar spiral lipid cylinders (‘cochleate cylinders’) that rearrange into large unilamellar vesicles only upon removal of calcium ions by EDTA (Papahadjopoulos et al., 1975; Kachar et al., 1986). Vesicles rapidly lose their contents in the course of these rearrangements. Calcium-induced fusion of vesicles of the synthetic lipid, sodium di-*N*-dodecylphosphate proceeds in a comparable way: small vesicles fuse into large flat vesicles, which rearrange into tubular calcium-lipid lamellar crystals (Rupert et al., 1987; Streefland et al., 1992; Fonteijn et al., 1992). However, for vesicles of many negatively charged lipids (including phosphatidylserine) and vesicles of mixtures of negatively charged and other lipids, there is ample evidence for the occurrence of non-leaky fusion of small vesicles into large vesicles (Wilschut et al., 1983; Hui et al., 1988). The molecular rearrangements proposed for such ‘tight’ calcium-induced fusion processes are similar to the stalk-pore hypothesis (Papahadjopoulos et al., 1990).

Cryogenic electron microscopy was chosen as a tool to examine the morphological changes of vesicles of DHPBNS and DDPBNS in the course of calcium-induced fusion. The experimental work described in this report required a compromise between optimal conditions for cryo-EM and limitations inherent in the model system of calcium-induced vesicle fusion. Cryo-EM would benefit from high lipid concentrations ( $> 5$  mM), large vesicle diameters (ca. 200 nm), and spherical symmetry, since in transmission view of a thin vitrified film, this would result in a neat coplanar arrangement of spheres in which interactions of adjacent bilayer membranes can be optimally resolved. On the other hand, selective exo-vesicular hydrolysis of the  $\beta$ -nitrostyrene lipids limits the

lipid concentration ( $< 2$  mM). Moreover, it was found that DHPBNS and DDPBNS do not yield perfectly spherical vesicles under all conditions, and binding of calcium ions leads to formation of very dense aggregates, which are not easily resolved. This report describes the morphology of lipid vesicles with an oligomerised inner leaflet and the morphological changes that are observed in the course of calcium-induced vesicle fusion. These lipid bilayer rearrangements are discussed within the context of recent views concerning the shape transformations and fusion of synthetic and biological membranes.

## 2. Materials and methods

### 2.1. Materials

DDPBNS and DHPBNS were prepared as described (Ravoo et al., 1996). Cholesterol was obtained from Sigma and *N*-octyl  $\beta$ -D-glucopyranoside was obtained from Aldrich. All other chemicals used were of analytical grade.

### 2.2. Vesicle preparation

Vesicles of DDPBNS and DHPBNS were prepared by three different methods. Small unilamellar vesicles were prepared by probe sonication and large unilamellar vesicles were prepared by extrusion through a 100 or 200 nm polycarbonate membrane, as described (Ravoo et al., 1999). In addition, large unilamellar vesicles of DHPBNS were prepared by detergent dialysis. A clear solution of mixed micelles of 5.0 mg DHPBNS and 30 mg *N*-octyl  $\beta$ -D-glucopyranoside in 1 ml of 5.0 mM HEPES/NaAc buffer (pH 7.4) was dialysed overnight against  $2 \times 100$  ml buffer incubated at 45°C. The buffer solution was replaced once, after ca. 3 h. Preparation of DHPBNS vesicles with an oligomerised inner leaflet was performed as described (with [DHPBNS] ca. 2 mM and pH 12) (Ravoo et al., 1996). Preparation of DDPBNS vesicles with an oligomerised inner leaflet was performed by a selective hydrolysis of the exo-vesicular BNS groups at 5°C with [DDPBNS] ca. 2 mM and pH 12 during 75 min, followed by

photopolymerisation of the endo-vesicular BNS groups during 10 min. These vesicles were kept at 5°C to avoid loss of bilayer asymmetry as a result of lipid flip-flop. These vesicles were used for EM on the day of preparation.

### 2.3. Calcium-induced fusion

Fusion experiments with vesicles of DHPBNS were performed with a lipid concentration of 1–2 mM in a 5.0 mM HEPES/NaAc (pH 7.4) buffer incubated at 50°C. Fusion was induced by the addition of 2.5 mM of  $\text{CaCl}_2$  while the mixture was stirred or vortexed. Samples for EM were taken after an incubation time of 30 s to 4 min. Fusion experiments with vesicles of DDPBNS with an oligomerised inner leaflet were performed with a lipid concentration of 1–2 mM in 5.0 mM HEPES/NaAc (pH 7.4) incubated at room temperature. Fusion was induced by the addition of 3.0–5.0 mM of  $\text{CaCl}_2$  while the mixture was stirred or vortexed. Samples for EM were taken after an incubation time of 0.5–3.0 min. Control experiments to study fusion of vesicles of DDPBNS were performed with a lipid concentration of 2.0 mM in 5.0 mM HEPES/NaAc (pH 7.4) incubated at room temperature. Fusion was induced by the addition of 10 mM  $\text{CaCl}_2$  while the mixture was stirred or vortexed. Samples for EM were taken after an incubation time of 30 s. In order to study the effect of EDTA addition after calcium-induced fusion of DDPBNS vesicles, samples that had been incubated with  $\text{CaCl}_2$  for 30 s were incubated with a four-fold excess (relative to calcium) of EDTA.

### 2.4. Electron microscopy

Samples for negative staining were prepared by conventional methods. Samples for cryo-EM were brought on a holey carbon coated grid (made hydrophilic by glow discharge in air), and a thin aqueous film was obtained by gentle blotting with filter paper. For comparison, several samples of DHPBNS vesicles were prepared in a Controlled Environment Vitrification System at 50°C and 100% relative humidity. The samples were vitrified by plunging them into liquid ethane. The grids

were quickly transferred to Gatan or Philips cryo-holders and examined in JEOL or Philips electron microscopes, operated at 100 or 120 kV. Images were recorded under low dose conditions at 0.5–1.0  $\mu\text{m}$  under focus.

## 3. Results and discussion

### 3.1. Morphology and calcium-induced fusion of DHPBNS vesicles

Negatively stained samples of sonicated vesicles of DHPBNS show 50–100 nm vesicles of spherical symmetry (Fig. 2). Either uranyl acetate or phosphotungstic acid can be used as staining agent. In the negatively stained preparations, the morphology and integrity of the vesicles do not appear to be affected by oligomerisation of the lipid head groups either in both leaflets or exclusively in the inner leaflet (Ravoo et al., 1996; Ravoo, 1998). Upon addition of calcium chloride (2.5 mM) to vesicles with an oligomerised inner leaflet, incubated above  $T_m$ , aggregates of 3–20 small vesicles are formed (Fig. 2). The aggregates become larger if either the calcium concentration or the incubation time is increased. It is impossible to establish from the negatively stained preparations whether these aggregates are either aggregates of intact vesicles with intact bilayers, or aggregates of vesicles that are hemifused and share a common bilayer diaphragm (Fig. 3). The latter conformation would represent an intermediate membrane fusion structure according to the stalk-pore model. Although some micrographs suggest this interpretation, they do not provide unambiguous evidence as a result of insufficient resolution of the bilayer.

Cryo-EM is able to resolve the bilayer structure of lipid vesicles, and some initial experiments were carried out using formvar/carbon coated grids as support for the vitrified sample solution. Again, addition of calcium chloride (2.5 mM) to sonicated vesicles with an oligomerised inner leaflet, incubated above  $T_m$ , resulted in the formation of small aggregates of vesicles (Fig. 4). The two leaflets composing the vesicle bilayer can clearly be discriminated as two separate lines, separated

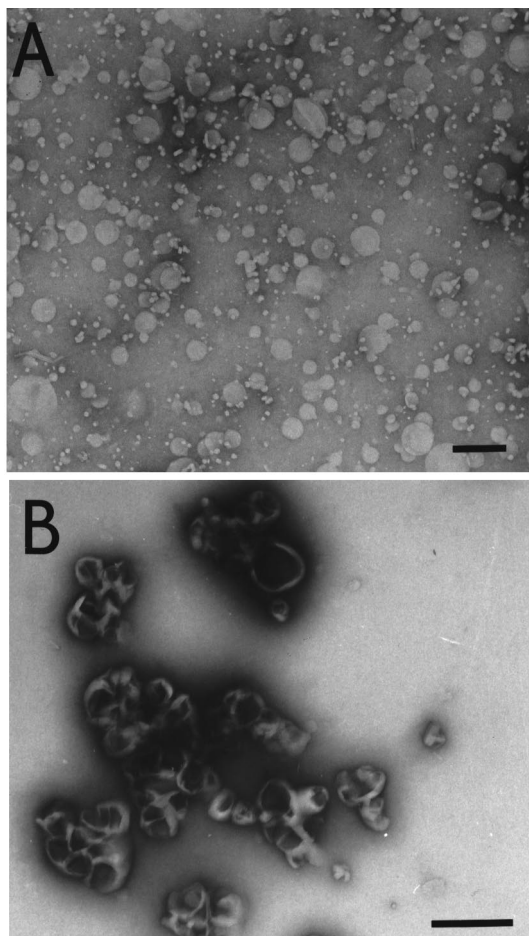


Fig. 2. Sonicated vesicles of DHPBNS (0.25 mM) with an oligomerised inner leaflet. Negative staining with PTA (A) and UAc (B). In (B), the vesicles were incubated for 4 min with  $\text{CaCl}_2$  (5.0 mM) at 50°C. Scale bar represents 500 nm.

by ca. 4 nm. Several of the aggregated vesicles share a common bilayer diaphragm. Possibly, this diaphragm is the result of hemifusion of these vesicles. We assume that the 'slow motion fusion' of the vesicles with an oligomerised inner leaflet (Ravoo et al., 1999) increases the probability of vitrification of this intermediate stage of the fusion process. In several cases, the diaphragm extends over more than 20 nm. The diaphragms which extend furthest, show signs of rupture, indicating instability of the arrested bilayer conformation. We note that occasional superposition,

instead of hemifusion, of two vesicles can also be observed in Fig. 4.

Admittedly, the preparation of cryo-EM samples on a formvar/carbon coated support film may suffer from possible artefacts arising from absorption of the vesicles to the surface of the support film. Therefore, Fig. 4 does not present unequivocal evidence of hemifusion of vesicles with an oligomerised inner leaflet.

Next, the samples were examined using vitrified aqueous films on holey carbon coated copper grids in order to exclude the influence of surface adsorption of the aggregated vesicles. We note that under these experimental conditions, the bilayers could only be resolved as a single dark line. Surprisingly, control experiments showed that vesicles of DHPBNS are flattened, ellipsoidal structures rather than spheres (Fig. 5). The atypical morphology of the vesicles was confirmed by observation of the samples under various tilt angles. The morphology was not affected by the method of preparation of the vesicles (sonication, extrusion, dialysis). In addition, the same flattened morphology was observed irrespective of whether vesicles were vitrified from a temperature above or below  $T_m$  (40°C), whether or not a Controlled Environment Vitrification System was used for sample preparation, whether or not the inner leaflet was oligomerised, and whether the vesicles were prepared either in buffer (with or without 140 mM of NaCl) or in water. Apparently, the morphology is inherent to the molecular structure of DHPBNS. This has also been observed for vesicles of several other synthetic lipids (Spevak et al., 1993; Hammarström et al., 1995; Zirkzee, 1997). Addition of 20 mol% of cholesterol to the vesicles improved the tendency of

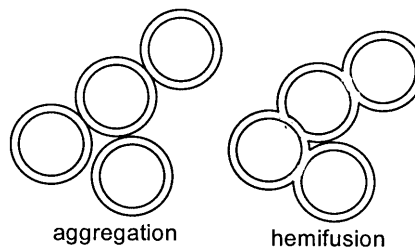


Fig. 3. Hypothetical transmission view of aggregated unilamellar vesicles and hemifused unilamellar vesicles.

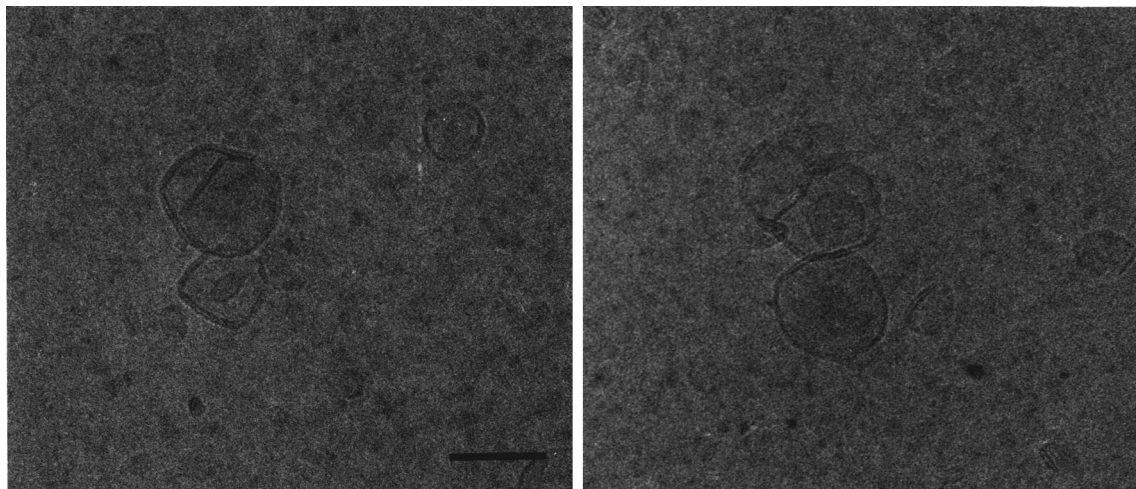


Fig. 4. Sonicated vesicles of DHPBNS with an oligomerised inner leaflet, incubated for 4 min with  $\text{CaCl}_2$  (2.5 mM) at 50°C. Cryo-EM on formvar/carbon support. Scale bar represents 100 nm.

DHPBNS to form spherical vesicles, but such an amount of cholesterol makes the bilayers stiff and most probably reduces their fusogenicity (Bentz and Ellens, 1988). The atypical morphology of DHPBNS vesicles can easily remain unnoticed in samples that are prepared on a formvar/carbon support, since most vesicles adsorb with their flattened side to the supporting film and appear spherical in projection (Fig. 2). However, the unsupported film yields a view of more random orientations of the vesicles, and indeed many vesicles are observed edge-on, or partially edge-on (Fig. 5). Also, many vesicles are bent, and even twisted.

Unfortunately, the irregular features of the flattened vesicles lead to an extremely complicated projection view. Aggregation and (hemi)fusion of such vesicles further increase the complexity, and it becomes difficult to discriminate bilayer aggregation and (hemi)fusion, and to check bilayer integrity. Therefore, the experiments were continued using DDPBNS, which has shorter hydrocarbon chains than DHPBNS, and is expected to yield more flexible, spherical bilayer vesicles.

### 3.2. Calcium-induced fusion of DDPBNS vesicles

Vesicles of DDPBNS display the anticipated smooth and spherical morphology when they are

examined by cryo-EM using a holey carbon-supported vitrified film of sample solution (Fig. 6). The bilayer is observed as a single dark line. As anticipated, the vesicles have a spherical morphology irrespective of their size and method of preparation, and the salt concentration of the aqueous medium. Occasionally, larger, multilamellar vesicles persist after sonication or extrusion. If vesicles are extruded through a 200 nm polycarbonate membrane, so-called 'vase-like' vesicles with large invaginations are frequently encountered (Lasic et al., 1997). In addition, the extruded vesicle solution contains relatively high numbers of small vesicles, which could reflect the relative ease of dispersion of DDPBNS in aqueous solution due to its rather short hydrophobic chains and relatively high water solubility.

First, the calcium-induced fusion of DDPBNS vesicles without oligomerised lipid was examined. Upon addition of calcium chloride (5 mM) to small or large vesicles of DDPBNS, aggregates with diameters up to 300 nm are formed within 30 s (EM data not shown). No remaining unilamellar vesicles can be detected. These aggregates have high density and occasionally a regular spacing of ca. 3 nm can be observed in the lipid packing. The dimensions of this spacing correspond to the width of a dodecyl lipid bilayer, but they are much too small for a stacking of hydrated lipid



bilayers at equilibrium distance, as in multilamellar vesicles. Possibly, the dense packing indicates almost completely dehydrated calcium-DDPBNS aggregates, as observed for phosphatidylserine (Papahadjopoulos et al., 1975). However, freeze-fracture EM or X-ray diffraction rather than cryo-EM should be used to determine the structure of these dense aggregates. In addition, the formation of completely dehydrated aggregates contradicts the retention of vesicle contents that has been observed during fusion of DDPBNS with DHPBNS vesicles (Ravoo et al., 1999). Upon addition of a four-fold excess of EDTA (relative to calcium) to the calcium-DDPBNS aggregates, they disperse within a few seconds and a solution of large unilamellar vesicles results (EM data not shown). The formation of large aggregates upon calcium addition, and the formation of large unilamellar vesicles upon EDTA addition are consistent with the quasi-elastic light scattering data reported previously (Ravoo et al., 1999).

The preparation of vesicles of DDPBNS with an oligomerised inner leaflet is feasible at 5°C, provided the selective hydrolysis of the exo-vesicular  $\beta$ -nitrostyrene groups of DDPBNS vesicles is rapidly followed by photopolymerisation of the remaining endo-vesicular  $\beta$ -nitrostyrene groups (Ravoo, 1998). The vesicle solution must be kept at low temperature to prevent loss of bilayer asymmetry as a result of lipid *flip-flop*. Using cryo-EM, it was observed that large vesicles of DDPBNS (prepared by extrusion through a 200 nm polycarbonate membrane) show a morpholog-

ical response to selective oligomerisation of the inner leaflet: they lose their smooth spherical appearance and show invaginations as well as protrusions (Fig. 6). Small vesicles (prepared by extrusion through a 100 nm membrane, or by sonication) do not show such a morphological response (Fig. 6). Possibly, the invaginations and protrusions are a consequence of the increased negative curvature of the inner leaflet as a result of oligomerisation of the head groups of the lipids in the inner leaflet. A similar curvature effect was observed in a monolayer study of DHPBNS (Ravoo, 1998) and discussed earlier (Ravoo et al., 1999), and has also been observed for comparable polymerisable lipids (Meier et al., 1994). We assume that monolayer destabilisation as a result of curvature strain affects the morphology of DDPBNS and not of DHPBNS bilayers because of the shorter hydrophobic chains that hold the bilayer together. Also, we assume that it affects large vesicles but not small ones, because the small ones cannot respond due to curvature restraints.

Upon addition of calcium chloride (2.5 mM) to small vesicles of DDPBNS with an oligomerised inner leaflet, the formation of aggregates of ca. 100 nm diameter was observed. These aggregates are much smaller than those observed upon addition of calcium chloride to small vesicles of DDPBNS without oligomerisation of the lipid head groups in the inner leaflet. Some aggregates comprise of only two or three vesicles. However, most aggregates were considerably larger and

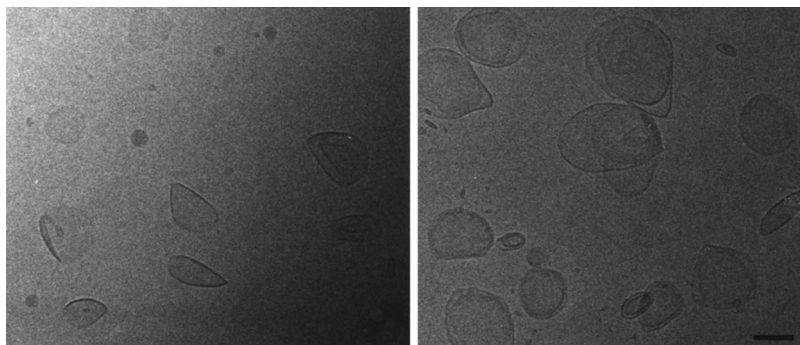


Fig. 5. Flattened and ellipsoidal vesicles of DHPBNS obtained by extrusion through 200 nm polycarbonate membranes. Cryo-EM on holey carbon films. Scale bar represents 100 nm.

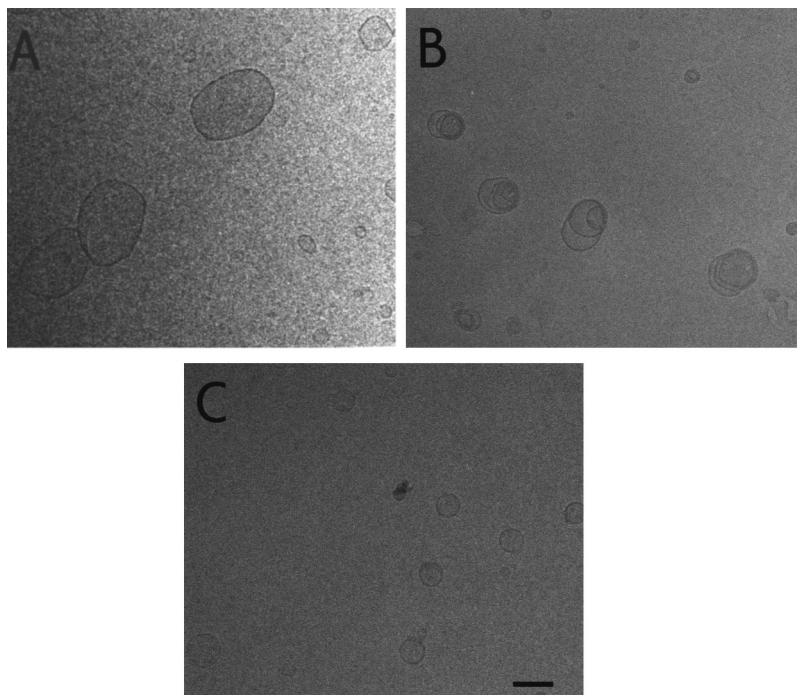


Fig. 6. Vesicles of DDPBNS. (A): Spherical vesicles obtained by extrusion through a 200 nm membrane. (B): Distorted vesicles obtained by extrusion through a 200 nm membrane, followed by oligomerisation of the inner leaflet. (C): Spherical vesicles obtained by extrusion through a 100 nm membrane, followed by oligomerisation of the inner leaflet. Cryo-EM on holey carbon films. Scale bar represents 200 nm.

denser, showing closely packed multilayer rims (Fig. 7). The aggregates become larger if either the calcium concentration or the incubation time is increased. The spacing of the rims is about 3 nm, as observed in the dense, calcium-induced aggregates of non-oligomerised vesicles. Upon addition of EDTA, the vesicles with an oligomerised inner leaflet behave very differently compared to the vesicles that are not oligomerised: the calcium-lipid aggregates disperse *slowly* into relatively small, *bilamellar* vesicles (Fig. 7). In addition, networks of thread-like, branched unilamellar vesicles were observed that extend over several microns (Fig. 7). These rearrangements occur on a time scale of several minutes. The thread-like vesicles appear to grow from the aggregates (Fig. 7). The width of the threads is variable, but no less than about 20 nm, as expected for two bilayers of charged lipids at equilibrium distance.

These experiments show that vesicles of DDPBNS fuse into dense aggregates upon addi-

tion of calcium chloride, but do so more slowly if they contain an oligomerised inner leaflet. In the latter case, fusing vesicles cannot be arrested in a hemifused intermediate stage, as was occasionally observed in the case of DHPBNS vesicles with an oligomerised inner leaflet. It is likely that the stability of hemifusion intermediates decreases with decreasing hydrophobic chain length of the lipid molecules (Siegel, 1993; Siegel, 1999).

Fig. 8 presents a speculative representation of the structural rearrangements that occur when first  $\text{CaCl}_2$  and then EDTA are added to vesicles of DDPBNS with an oligomerised inner leaflet. Upon addition of  $\text{CaCl}_2$ , vesicles fuse into multilamellar aggregates. The vesicles fuse faster and more extensively at higher concentrations of  $\text{CaCl}_2$ , in accordance with the results of lipid mixing assays (Ravoo, 1998; Ravoo et al., 1999). The aggregates are largely dehydrated as a result of efficient binding of calcium ion to the DDPBNS head groups, which is reflected by the

close packing of the lamellae. At present, whether or not the asymmetry of the fusing bilayers remains intact in the multilamellar arrangement is uncertain. The aggregates slowly disperse upon addition of EDTA, and one could imagine that a kinetic phase separation of monomer and oligomerised DDPBNS occurs at this stage, simply because the monomers will rearrange much faster than the oligomers. Perhaps the monomers end up in the bilamellar vesicles, whereas the

oligomers build up the thread-like vesicles. The thread-like vesicle morphology could be the result of the preference of the linear lipid oligomers for a parallel orientation upon dispersal of the calcium-lipid aggregates. However, the mechanism of formation of the bilamellar vesicles is unclear. It is unlikely that the bilamellar vesicles are a result of shear-induced break-up of the network of thread-like vesicles, since this should produce unilamellar vesicles. Finally, we note that these

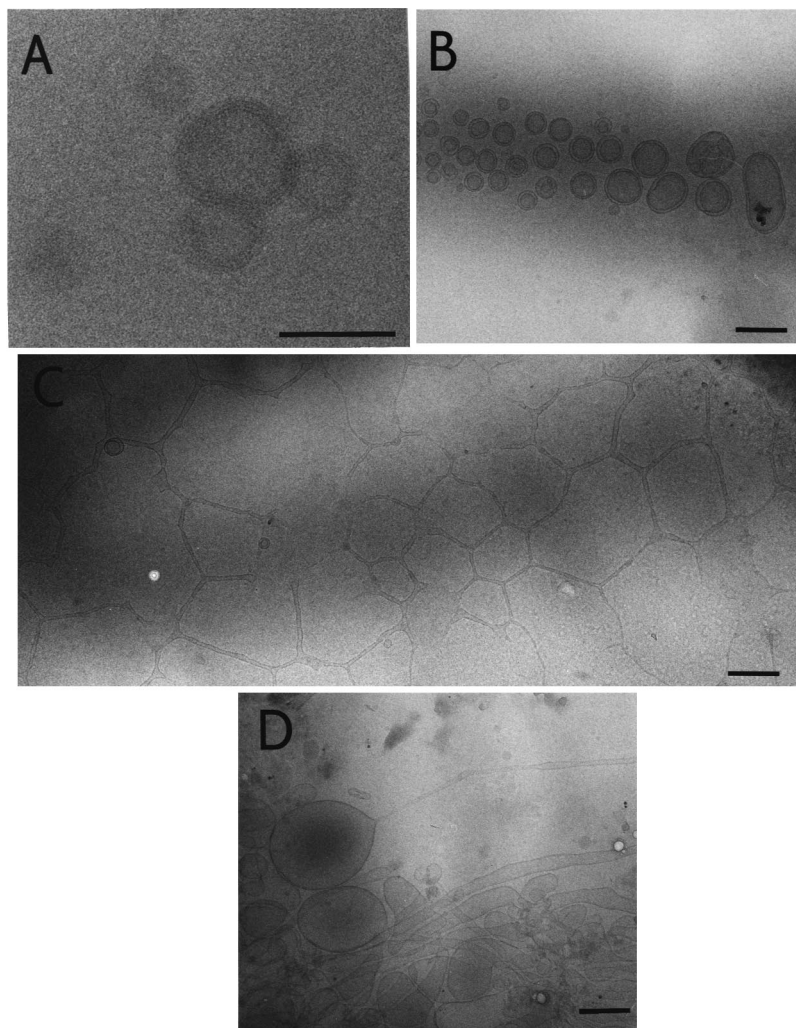


Fig. 7. Calcium-induced fusion of sonicated vesicles of DDPBNS with an oligomerised inner leaflet. (A): Vesicles of DDPBNS (2.0 mM), incubated with 3.0 mM of  $\text{CaCl}_2$  for 3 min. (B), (C) and (D): Vesicles of DDPBNS (2.0 mM) incubated with 5.0 mM of  $\text{CaCl}_2$  for 30 s, then incubated with 25 mM of EDTA for 30 s. Cryo-EM on holey carbon films. Scale bar represents 100 nm in (A) and 200 nm in (B), (C) and (D).

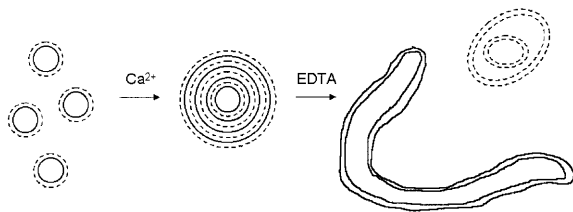


Fig. 8. Cartoon of the structural rearrangements of DDPBNS vesicles with an oligomerised inner leaflet upon addition of  $\text{CaCl}_2$ , followed by EDTA. Dotted lines represent monomer DDPBNS, solid lines represent oligomerised DDPBNS. The multilamellar arrangement in the  $\text{Ca}^{2+}$ -DDPBNS aggregate is uncertain.

extensive rearrangements upon addition of EDTA to the calcium-lipid aggregates can account for the additional lipid mixing that has been observed under these conditions (Ravoo et al., 1999). This additional lipid mixing was not observed if the parent vesicles did not contain an oligomerised inner leaflet.

In summary, vesicles of DHPBNS are flattened, ellipsoidal structures and the analysis of their interactions is complicated. Electron microscopic evidence was obtained for arrested calcium-induced hemifusion of DHPBNS vesicles with an oligomerised inner leaflet, but the evidence is not fully conclusive. Compared with vesicles that are devoid of oligomerised lipids, vesicles of DDPBNS with an oligomerised inner leaflet show slow but complicated morphological changes during calcium-induced fusion, as well as upon quenching of the fusion process with EDTA.

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